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Running title: Dechlorane 602 and immune responses in mice

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Abstract

Background: Although the chlorinated flame retardant Dechlorane (Dec) 602 has been detected in food, human blood and breast milk, there is limited information on potential health effects, including possible immunotoxicity.

Objectives: To determine the immunotoxic potential of Dec 602 in mice by examining the expression of phenotypic markers on thymocyte and splenic lymphocyte subsets, Th1/Th2 transcription factors, and the production of cytokines and antibodies.

Methods: Adult maleC57BL/6 mice were orally exposed to environmentally relevant doses of Dec 602 (1, 10 μg $kg^{-1}$of body weight per day) for 7 consecutive days. Thymocytes and splenic CD4 and CD8 subsets, and splenocyte apoptosis were examined by flow cytometric analysis. Cytokine expression was measured at both mRNA and protein levels. Th1 (T-bet and STAT1) and Th2 (GATA3) transcriptional factors were determined using qPCR. Serum levels of IgG_1, IgG_{2a}, IgG_{2b} and IgE were measured using ELISA.

Results: Splenic $CD4^+$ and $CD8^+$ T cell subsets were decreased compared with vehicle controls, and apoptosis was significantly increased in splenic $CD4^+$ T cells. Cytokine expression (mRNA and protein) showed increases in Th2 cytokines (IL-4, IL-10, and IL-13), and decreases in Th1 cytokines (IL-2, IFN-γ and TNF-α). Th2 transcriptional factor GATA3 was increased while Th1 transcriptional factors T-bet and STAT1 were decreased. As additional indicators of Th2-Th1 imbalance, production of IgG_1 was significantly increased, while IgG_{2a} was reduced.

Conclusions: To our knowledge, we are the first to report evidence of effects of Dec 602 on immune function in mice, with findings indicating that Dec 602 exposure favored Th2 responses and reduced Th1 function.

Introduction

Dechlorane, or Mirex, is a chlorinated flame retardant that was widely used before concerns about environmental effects prompted its replacement with analogs such as Dechlorane plus (DP), Dechlorane (Dec) 602, 603, and 604 (Sverko et al. 2011). However, these chemicals have also been detected in the environment in recent years, The environmental occurrence of Dec 602, 603 and 604 was first reported in 2009, and it is anticipated that they are persistent and bioaccumulative in the environment because of their high degree of chlorination (Shen et al. 2009). Little information is available on the production of Dec 602. It is listed on the Canada's Nondomestic Substances List and the European Chemical Substances Information System which indicate that Dec 602 is currently in use (Shen et al. 2011). Dec 602 is being used in fiberglass-reinforced nylon-6 at 18% (Chanda and Roy 2006).

Dec 602 has been reported in various environmental media, possibly because it is more bioaccumulative than DP and other chlorinated flame retardants that are currently being used (Dec 603 and Dec 604). Dec 602 concentration in fish was about 15-80 times higher than DP, and the bioconcentration factors (BCF) of Dec 602 is about 40 times higher than DP (Shen et al. 2009). A study conducted in Northern China further also confirmed that the biota-sediment accumulation factor (BSAF), a measure of the bioaccumulation potential of hydrophobic organic compounds in aquatic biota, is higher for Dec 602 than DP (Jia et al. 2011). Several studies suggest that Dec 602 could be a global environmental contaminant. As an example of biota from a remote environment, Dec 602 was detected in all samples of arctic beluga, while other types of

dechloranes were not detected (Shen et al. 2012). Dec 602 has also been detected in air and seawater (Xian et al. 2011). Dec 602 detection in the environment suggests that humans are at a constant risk of Dec 602 exposure. Other chlorinated flame retardants such as Mirex have been banned due to evidence of adverse health effects, including endocrine, immune, developmental, and reproductive toxicity (de Cock and van de Bor 2014). However, to our knowledge, no toxicity studies of Dec 602 on animals have been reported. Therefore, there is an urgent need to test the toxicity of this commonly used product.

Immune system has been considered as a sensitive target when exposed to environmental pollutants (Inadera 2006). Studies have shown that persistent organic pollutants (POPs) and polybrominated diphenyl ethers (PBDEs) can exert acute toxic effects on the immune system (Fernandez-Salguero et al. 1995; Leijs et al. 2009). It was hypothesized that Dec 602 could target acquired immune responses and cause immune dysregulation (e.g., Th1/Th2 imbalance) following a short-term exposure. In this study, the effects of Dec 602 on immune system, especially T cells in mice that had been exposed systematically at 1 and 10 μg $kg^{-1}$of body weight per day were studied, which provided us new insights into understanding potential adverse effects of Dechloranes and other chlorinated flame retardants.

Methods

Animals

Male C57BL/6 mice (6 weeks old, 20±1g) were purchased from Vital River Laboratories (VRL; Beijing, China) and housed in the Animal Research Center of Tsinghua University under specific pathogen-free conditions, at a controlled temperature of 24±2°C and humidity of 50%±10%, with a cycle of 12 hours light and 12 hours dark. Animals were provided *ad libitum* pellet feeds and water, and randomly assigned into three groups. The mice in two treated groups were administered with Dec 602 (Toronto Research Chemicals Inc.) in olive oil (low: 1, high: 10μg $kg^{-1}$of bodyweight per day) by gavage. Controls received olive oil only.

The exposure doses used in the present study were selected to represent environmental exposures, based on the following information. It is reported that Dec 602 concentration in sediments was 6 ng/g dry weight, and this concentration has accumulated to 34 ng/g lipid in fish from the same area(Shen et al. 2009). Given the higher BCF and bioavailability of Dec 602, it is reasonable to suggest that Dec 602 concentrations are amplified along the food web (Shen et al. 2009). The level of lipid in fish from the Great Lakes was up to 9.0g lipid/100g fish. Thus, the concentration of Dec 602 in fish was up to 3 ng/g fish (Turyk et al. 2012). Based on the concentration in fish, environmentally relevant doses of 1 and 10 μg kg^{-1}·bw (or 1 and 10 ng g^{-1}·bw) were used in the current mouse study. After 7 days exposure, the bodyweights were measured, sera were collected immediately after euthanasia, and thymus and spleen tissues were

weighed and processed for further studies. All animal protocols were approved by the Animal Care and Use Committee of Tsinghua University and performed humanely for alleviation of suffering.

Histopathological examinations

Organs were collected and fixed in 2.5% (v/v) glutaraldehyde-polyoxymethylene solution immediately after euthanasia. The tissue samples were dehydrated and embedded in paraffin wax. Serial paraffin sections (4μm) were obtained and kept at 37°C for more than 12 hours. The sections were immersed in three consecutive xylene washes for 5 min each to remove paraffin and subsequently hydrated with 5 consecutive ethanol washes in descending order: 100%, 95%, 80%, 70%, and deionized water. The paraffin sections were then treated with Hematoxylin-eosin (H&E) staining, and changes in organizational structure were visualized using a light microscope with Leica Application Suite software (Leica Microsystems, Wetzlar). Five sections were examined from each animal and 5 animals were evaluated for each group.

Flow cytometry

Spleen and thymus were harvested, and single cell suspensions were prepared by homogenizing on 70μm cell strainers (BD Falcon) using PBS with 2% FBS. Spleen red blood cells were removed at 37^{o}C by adding 2ml of 1x lysing buffer (BD Biosciences) for 3 minutes. Cells were washed twice with cold PBS, and stained for 30 minutes in an appropriately diluted antibody staining solution. Antibodies used were: rat anti-mouse CD3-Percp-Cy^{TM}5.5, rat anti-mouse

CD4-FITC and rat anti-mouse CD8-PE-Cy^{TM}7, and isotype controls were Percp-Cy^{TM}5.5 Rat IgG_{2a}, FITC Rat IgG_{2a} and PE-Cy^{TM}7 Rat IgG_{2a}, respectively. For each 10^6 cells, 0.5μg antibody was used for staining. Antibodies were purchased from BD (BD Biosciences).

PE-Annexin V and 7-amino-actinomycin D (7-ADD) were employed for the apoptosis analysis. Annexins are a family of intracellular proteins that bind to phosphatidylserine (PS). PS translocates to the outer membrane leaflet upon initiation of apoptosis. Necrotic or dead cells with increased membrane permeability are stained with 7-ADD. Thus, Annexin V single positive represents the early apoptotic stage of cells and Annexin V/7-ADD double positive indicates late stage of apoptosis. Cells were resuspended in 1x binding buffer and stained with PE-Annexin V and 7-ADD for 15 minutes at room temperature. Samples with a final volume of 400μl were tested within 1 hour and a total of 5000 events were collected. $CD4^+$ and $CD8^+$ cells were first gated, and apoptotic cells were analyzed within $CD4^+$ and $CD8^+$ cell subsets. Data were collected on BD LSRII (BD Biosciences) and analyzed using Cell Quest software.

RNA isolation and quantitative real-time polymerase chain reaction (qPCR)

Total RNA was purified from spleen tissue using TRIzol reagent (Invitrogen), and 2 μg of RNA were reversed transcribed using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). qPCR of IL-4, IL-10, IL-13, IFN-γ, TNF-α, IL-2, T-bet, STAT1 and GATA3 were performed on equal amounts of cDNA, using GoTaq qPCR Master Mix (Promega). The housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT) was used as an internal standard. The

SYBR green signal was detected by LightCycler®480 Instrument (Roche). The relative transcript expression levels were quantified using the ΔΔCt method (Winer et al. 1999). The specificity of amplification was confirmed by melting curves and by gel electrophoresis.

Luminex assay

The concentrations of selected cytokines (IL-4, IL-10, IL-13, IFN-γ, TNF-α, and IL-2) in mouse sera and spleen protein extracts obtained using NP40 cell lysis buffer (Life Technologies) were determined by Luminex assay with a custom kit, the MCYTOMAG-70KBead Panel (EMD Millipore Corporation). The instrument used was Luminex 200^{TM} (Luminex) and read under Mag Plex. Data were analyzed using Milliplex Analyst 5.1 Software (Merck Millipore). All samples were tested in duplicate for Luminex assay.

ELISA

Levels of serum IgG_1, IgG_{2a}, IgG_{2b} and IgE were measured using ELISA kits purchased from eBioscience (San Diego, CA). Briefly, capture antibody (1:1000 v/v) was coated separately on microtiter plates, incubated overnight at 4°C. After washing with PBS containing 0.05% Tween-20 (PBST), microplate wells were blocked with 5% BSA at room temperature for 2 h. After washing with PBST, diluted samples were added to the microplate and incubated for 2 h at room temperature. After washing with PBST, HRP-conjugated detection antibody was added to each well and incubated for 1 h at room temperature. After washing with PBST, TMB was added and incubated for 15 min at room temperature. The enzyme reaction was stopped with 1 M H_3PO_4.

The optical density was read using a microplate fluorescence reader (Tecan Infinite F200 Pro) at a wavelength of 450 nm.

Statistical analysis

Experimental data were analyzed by one-way ANOVA or Dunnett test using GraphPad Prism 5 (GraphPad Software). Results are presented as mean ± SD. Differences were classified as significant * for $p<0.05$, ** for $p<0.01$ and *** for $p<0.001$ from control.

Results

Body weights, relative organ weights, and thymocyte subsets following Dec 602 exposure

Exposure to Dec 602 had no significant effects on body weight, organ weights, and relative organ weights of brain, liver, thymus, spleen and kidneys (Table 1). In addition, histopathological examination was conducted, and the results obtained by H&E staining showed no notable changes in spleen and liver (Figure1A-1D). To study the effects of Dec602 on T cell differentiation, thymocytes were collected following 7 days of exposure. Immature double positive (DP) $CD4^+CD8^+$, mature single positive (SP) $CD4^+$ and SP $CD8^+$ thymocyte subsets were tested by FACS. Thymocyte subsets showed no significant changes after Dec 602 exposure (Figure 2A-2D).

Differential splenic T cell subtypes

To further test the effects of Dec 602 on the overall immune status, splenic T cell differentials were examined using FACS. When compared to control group, $CD3^+$ T cells were significantly lowered in the 10μg kg^{-1}·bw treatment group (Figure 3A and 3D). No significant change of $CD3^+$ cells was found in the 1μg kg^{-1}·bw group. Subtypes of T cells were also tested. Similar changes occurred in the $CD3^+CD4^+$ T cell population where it was significantly decreased in the 10μg kg^{-1}·bw group (Figure 3B and 3E; $p<0.05$). $CD3^+CD8^+$ lymphocytes showed significant reductions (Figure 3C and 3F; $p<0.001$) in both Dec 602-treated groups, indicating a possible impairment of cytotoxic T lymphocyte function.

Spleen T cell apoptosis

Above results suggested that both $CD4^+$ and $CD8^+$ T cell subsets were decreased significantly in spleen following Dec602 exposure. To examine whether apoptosis was induced by Dec 602 in the 10 μg kg^{-1}·bw group, splenocytes were stained with apoptosis markers Annexin V and 7-ADD and determined by FACS (Figure 4A). Results showed that early apoptotic $CD4^+$ T cells (Annexin V^+ and 7-ADD^-) were increased significantly ($p<0.05$) after Dec602 exposure (Figure 4B left panel), while no difference was found in late apoptotic cells (Annexin V^+ and 7-ADD^+; Figure 4B right panel). We also observed non-significant ($p > 0.05$) increases in early and late apoptotic $CD8^+$ T cells (increases of 1.25 ± 1.42% and 2.18 ± 2.56%, respectively) (Figure 4C).

Th2 and Th1 cytokine expression

Cytokines of both Th1 and Th2 were tested following Dec 602 exposure at the mRNA level in spleen using qPCR, and at protein level in sera and spleen protein extracts using Luminex assay. Typical Th2 cytokines including IL-10, IL-13 and IL-4 were examined, and significant increases were found for all these cytokines at the mRNA level (Figure 5A-5C). The IL-10 mRNA (Figure 5A) was 1.73 ± 0.45 times higher ($p<0.01$) in the 10μg kg^{-1}·bw group. IL-4 (Figure 5B) and IL-13 (Figure 5C) mRNAs were also increased significantly. In the 1 μg kg^{-1}.bw treatment group, we observed non-significant increase in IL-4 (2.07 ± 0.62 times) (Figure 5B) and IL-13 (1.85±0.36 times) (Figure 5C), while in the 10μg kg^{-1}·bw group the folds of increase were 4.29±1.49 times ($p<0.01$) and 3.98±1.88 times ($p<0.05$), respectively. IL-10 protein level in sera was increased significantly in the 10μg kg^{-1}·bw treatment group (Figure 5D; $p<0.05$) while IL-4and IL-13 were not significantly different from controls (Figure 5E, 5F). Both IL-10 and IL-4 levels from spleen protein extracts were increased significantly in the 10 μg kg^{-1}·bw treatment group (Figure 5G; $p<0.05$; Figure 5H;$p<0.01$).

For Th1 cytokines, the mRNA levels of IFN-γ (Figure 6A), TNF-α (Figure 6B) and IL-2 (Figure 6C) were decreased significantly in the 10μg kg^{-1}·bw treatment group ($p<0.05$). No obvious decreases were seen in the 1μg kg^{-1}·bw treatment group. Luminex analysis of sera showed a significant decrease of IL-2 at protein level in the 10μg kg^{-1}·bw treatment group (Figure 6F; $p<0.05$). However, IFN-γ and TNF-α levels in both sera and spleen protein extracts showed no significant differences from controls in either treatment group (Figure 6D, 6E, 6G, 6H).

Th2 and Th1 transcription factors

Patterns of cytokine expression suggested a shift in the Th1/Th2 balance after Dec 602 exposure. Therefore, we examined the expression of transcriptional factors for Th1 (T-bet, STAT1) and Th2 (GATA3) cells in the spleen using qPCR. We found that T-bet and STAT1 were decreased significantly in both Dec 602 treatment groups (Figure 7A and 7C; $p<0.01$ and $p<0.001$) while GATA3 was increased significantly in the 10μg kg^{-1}·bw group (Figure 7B; $p<0.05$).

Serum IgG_1, IgG_{2a}, IgG_{2b}, and IgE levels

Th1 cells induce production of IgG_{2a} antibody and Th2 cells promote IgE and IgG_1 (Peng et al. 2002). IgG_{2b} can be either Th1 or Th2 response under different conditions (Bennett J. 2003, Dixit et al. 2014). Thus, serum antibody levels of IgG_1, IgG_{2a}, IgG_{2b} and IgE were tested using ELISA. The level of IgG_1 (Figure 8A) was increased by 58±2%in the 1μg kg^{-1}·bw group ($p<0.01$) and by 47±2% in the 10μg kg^{-1}·bw group ($p<0.05$), respectively. IgG_{2a} (Figure 8B) showed a significant decrease of 51±3% in the 10μg kg^{-1}·bw group ($p<0.01$). There were no significant differences in IgE or IgG_{2b} production for either treatment group relative to controls (Figure 8C and 8D).

Discussion

Although dechloranes have been introduced into the environment for decades, there is limited information about their potential toxicity. Previous studies regarding DP toxicity used relatively high doses, e.g. 5×10^5, 2×10^6, and 5×10^6 μg kg^{-1}·bw for 10 days (Wu et al. 2012) and 10^3, 10^4 and 10^5μg kg^{-1}·bw for 90 days (Li et al. 2013), and these studies showed undetectable or low

toxicity of DP. However, considering the daily usage, low dose effects of DP as well as other isomers should be conducted (Zhu et al. 2007). After 7 consecutive days of relatively low dose Dec 602 exposure, our studies showed decreased percentages of $CD4^+$ as well as $CD8^+$ T cell subsets in spleen. Several reasons could lead to changing profile of T cells, and here, we showed splenic T cell apoptosis might be one of the underlying mechanisms.

T cells regulate immune responses by secreting cytokines. IFN-γ secretion by Th1 cells inhibits Th2 cell proliferation, while IL-4 secretion by Th2 cells can inhibit Th1 proliferation. In addition, the cytokine IL-10 modulates the Th1/Th2 balance in favor of Th2 cell responses by inhibiting the production of IFN-γ by Th1 lymphocytes (Asadullah et al. 2003). Dec 602 exposure at 10 $\mu g\ kg^{-1}\cdot bw$ caused increased IL-10 and IL-4 mRNA expression, and decreased IFN-γ expression. This pattern of cytokine expression is consistent with a shift in favor of Th2 cell proliferation. Additional studies on the expression of transcriptional factors of both Th1 and Th2 also support Dec 602 caused Th1/Th2 dysregulation. STAT1 pathway is one of the signal transduction pathways controlling T-bet expression (Afkarian et al. 2002). The mRNA level of STAT1 was significantly decreased following Dec 602 exposure, suggesting possible involvement of IFN-γ/STAT1 pathway. IgG_1 and IgG_{2a} production can reflect Th1/Th2 reactivity. Importantly, IgG_1 increased while IgG_{2a} decreased, consistent with an increase in Th2 and decrease in Th1 responses, respectively. Increased ratios of GATA3/T-bet and IgG_1/IgG_{2a}, together with the pattern of cytokine expression suggested that Th1/Th2 balance was shifted to

the Th2 phenotype, i.e., that T cell differentiation may be modulated by Dec 602. Dysregulation of the Th1/Th2 balance may alter cytokine patterns and resulting aberrant immune responses.

One of the reported mechanisms for tumor escape is that recruited immune cells express cytokines such as IL-13 and IL-4 to generate a tumor-favoring microenvironment. In addition, high concentrations of TNF-α has antitumor effects while constant low expression of TNF-α can induce a tumor phenotype (van Horssen et al. 2006). IL-2 plays important roles in maintaining T cell activities, including anti-tumor activity. IL-2 administration is an effective cancer immunotherapy (Rosenberg 2014). Mitogen-activated protein kinase (MAPK) pathways have critical roles in production of both IL-2 and TNF-α (Chi et al. 2006; Li et al. 1999). Dec 602 may negatively regulate MAPK activity, and further study should be conducted to verify molecular mechanisms in Dec 602-induced dysregulation of both cytokines. In the present study, we observed increased IL-13, IL-4 and IL-10 levels and decreased IL-2, IFN-γ, and TNF-α after Dec 602 exposure, which supports the need for additional research to determine whether exposure might promote a dysregulated immune environment that could increase vulnerability to tumor evasion (Vesely et al. 2011). This situation may be further exacerbated by decreased IFN-γ levels in mice exposed to Dec 602 (Zhang et al. 2008).

Dec 602 has been identified in dietary products such as meat and fish at various concentrations (Kim et al. 2014). 50 percent sera from Norwegian women contained measurable Dec 602 (Cequier et al. 2015). In another study, Dec 602 has been detected in human sera and breast milk collected from approximately 100 Canadian women during 2007–2009 (the maximum value in

sera was up to 5.7 ng/g lipid and the median concentrations were 0.53 and 0.23 ng/g lipid in sera and breast milk, respectively), suggesting a potential of mother-to-fetus transmission (Zhou et al. 2014). In our study of adult male mice, short-term and low dose exposure to Dec 602 appeared to alter the Th1-Th2 balance. However, responses to Dec 602 may differ between newborns and adults (as well as between humans and mice), and therefore further experiments are needed to evaluate differences in effects of Dec 602 according to developmental stage.

Murine models have long been used to closely mimic human inflammatory conditions (Takao and Miyakawa 2015). If Dec 602 is a potent immunosuppressive chemical in the mouse, it is likely that it would be immunotoxic to humans as well. However, there are major differences in both the innate and cellular immune responses of mice and humans (Mestas and Hughes 2004), and future studies should also use human cells to investigate the immunotoxic effects of Dec 602. To our knowledge, no one has conducted the experiment to measure background Dec 602 levels in the serum of experimental animals nor the serum level of Dec 602 in animal experiment after Dec 602 exposure. We are setting up a credible and feasible method to determine the serum concentration of Dec 602 in mice, and it is our intention to further investigate the serum and tissue concentrations of Dec 602 in mice.

In summary, our findings indicated that short-term Dec 602 exposure altered immune responses in adult male mice. Th1 suppression may result in attenuated host resistance that makes the body more vulnerable to infections and cancer (Ilinskaya and Dobrovolskaia 2014). On the other hand, enhanced Th2 response can trigger airway hyper responsiveness (Wills-Karp et al. 1998),

resulting in potential risk of allergic asthma. Given the immunotoxic effects of Dec 602, further studies using various animal models to determine cancer risks and asthma are warranted.

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Table 1.Body weight and organ weights following Dec 602 exposure

Parameters	0	1μg	10μg
BW(g)	18.83±1.31	18.96±1.33	18.41±2.11
Brain(g)	0.41±0.02	0.40±0.02	0.41±0.03
Liver(g)	1.04±0.18	1.04±0.14	0.98±0.12
Thymus(g)	0.06±0.01	0.06±0.01	0.06±0.01
Spleen(g)	0.06±0.01	0.06±0.01	0.06±0.01
Kidneys (g)	0.27±0.02	0.27±0.02	0.27±0.02
Brain(%BW)	2.17±0.15	2.12±0.17	2.27±0.45
Liver(%BW)	5.48±0.69	5.50±0.52	5.44±1.44
Thymus(%BW)	0.36±0.10	0.35±0.07	0.41±0.20
Spleen(%BW)	0.32±0.05	0.30±0.04	0.33±0.06
Kidney(%BW)	1.46±0.07	1.41±0.07	1.45±0.25

n=5-8.

Note: 1μg and 10 μg represent 1μg kg^{-1}·bw and10μg kg^{-1}·bw, respectively.

Figure legends

Figure 1. A, B, histopathological studies of spleen; C, D, histopathological studies of liver. Scale Bar = 50 μM. Sections were stained with H&E, and images shown in the figure are representatives of all the sections examined.1μg and 10 μg represent 1μg kg^{-1}·bw and10μg kg^{-1}·bw.

Figure 2. Effect of Dec 602 thymocyte differentiation. $CD4^+$ and $CD8^+$ T lymphocytes were tested using FACS as described. A, a representative dot plot of CD4 vs. CD8; B, %SP $CD4^+$ thymocytes; C, %SP $CD8^+$ thymocytes; and D, %DP $CD4^+CD8^+$ thymocytes. n = 5-8.X-axis labels：1μg and 10 μg represent 1μg kg^{-1}·bw and10μg kg^{-1}·bw, respectively. Values shown in bar graphs are means ± SD.

Figure 3. Dec 602 affected T cell development in spleen. $CD3^+$, $CD4^+$ and $CD8^+$ T lymphocytes were tested using FACS as described. A, B and C, representative dot plots of CD3, CD4 and CD8, respectively; D,%$CD3^+$ lymphocytes; E,%$CD4^+$ lymphocytes; F,%$CD8^+$ lymphocytes. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$. n = 5-8. X-axis labels：1μg and 10 μg represent 1μg kg^{-1}·bw and10 μg kg^{-1}·bw, respectively. Values shown in bar graphs are means ± SD.

Figure 4. Dec 602 affected splenic T lymphocyte apoptosis. A, $CD4^+$ and $CD8^+$ lymphocytes were gated and cell apoptosis was analyzed within $CD4^+$ and $CD8^+$ lymphocytes. Numbers in upper right and lower right quadrants represent end stage (Annexin V^+ and 7-ADD^+) and early stage apoptosis (Annexin V^+ and 7-ADD^-), respectively; B, early and end stage apoptosis in

$CD4^+$ T lymphocyte subset; C, early and end stage apoptosis in $CD8^+$ cell subsets.*, $p<0.05$. n = 5-8. X-axis labels： 1μg and 10 μg represent 1μg kg^{-1}·bw and10 μg kg^{-1}·bw, respectively. Values shown in bar graphs are means ± SD.

Figure 5. The mRNA levels of Th2 cytokines in spleen and their protein levels in sera and spleen. Cytokines were examined at mRNA levels using qPCR, and at protein levels in sera and spleen using Luminex as described. A, B, C, mRNA for IL-10, IL-4and IL-13, respectively; D,E,F, protein levels for IL-10, IL-4and IL-13 in sera, respectively; G, H ,protein levels for IL-10 and IL-4in spleen extracts, respectively.*, $p< 0.05$, **, $p< 0.01$. n = 5-8. X-axis labels： 1μg and 10 μg represent 1μg kg^{-1}·bw and10 μg kg^{-1}·bw, respectively. Values shown in bar graphs are means ± SD.

Figure 6. The mRNA levels of Th1 cytokines in spleen and their protein levels in sera and spleen. Cytokines were examined at mRNA levels using qPCR, and at protein levels in sera and spleen using Luminex as described. A, B, C, mRNA for IFN-γ, TNF-α and IL-2, respectively; D,E,F, protein levels for IFN-γ, TNF-α and IL-2, in sera, respectively; G, H, protein levels for IFN-γ and TNF-αin spleen extracts, respectively.*, $p< 0.05$. n = 5-8. X-axis labels： 1μg and 10 μg represent 1μg kg^{-1}·bw and10 μg kg^{-1}·bw, respectively. Values shown in bar graphs are means ± SD.

Figure 7. Effects of Dec 602 on Th1/Th2 transcriptional factor expression. T-bet (Th1; A) as well as the key Th1 regulator STAT1 (C) and GATA3 (Th2; B) were tested at mRNA level using

qPCR as described. Bars represent the mean ± SD. *, $p< 0.05$, **, $p< 0.01$, ***, $p< 0.001$. n = 3-5. X-axis labels：1μg and 10 μg represent 1μg kg^{-1}·bw and10 μg kg^{-1}·bw, respectively. Values shown in bar graphs are means ± SD.

Figure 8. Effects of Dec 602 on serum levels of IgG subclasses and IgE. ELISA was performed as described. Bars represent the mean ± SD. A,IgG_1; B, IgG_{2a}; C, IgG_{2b}; D, IgE. *, $p< 0.05$, **, $p< 0.01$. n = 5-8. X-axis labels：1μg and 10 μg represent 1μg kg^{-1}·bw and10 μg kg^{-1}·bw, respectively. Values shown in bar graphs are means ± SD.

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Figure 1

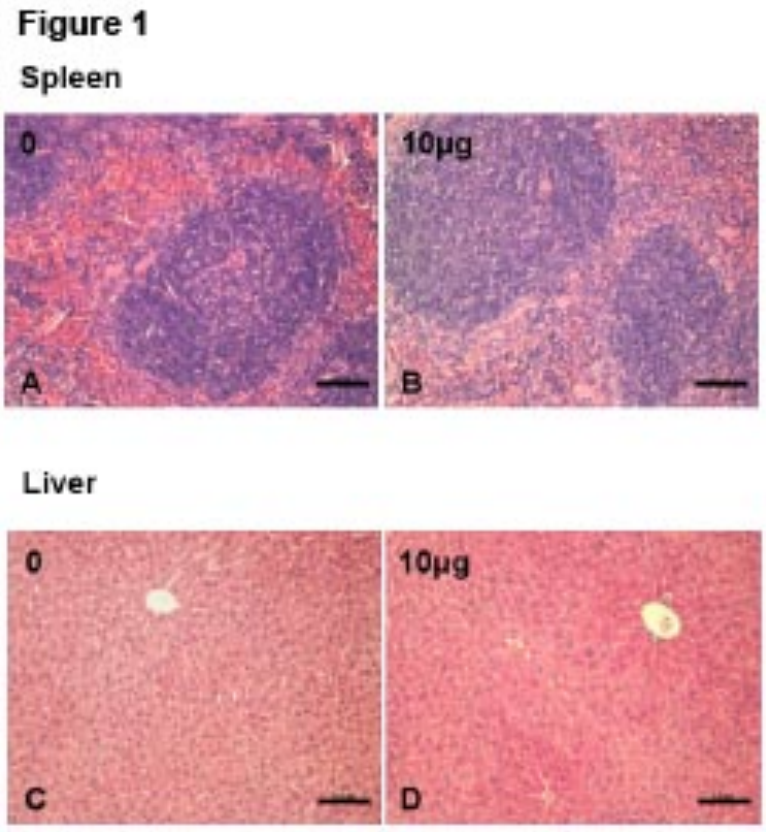

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Figure 2

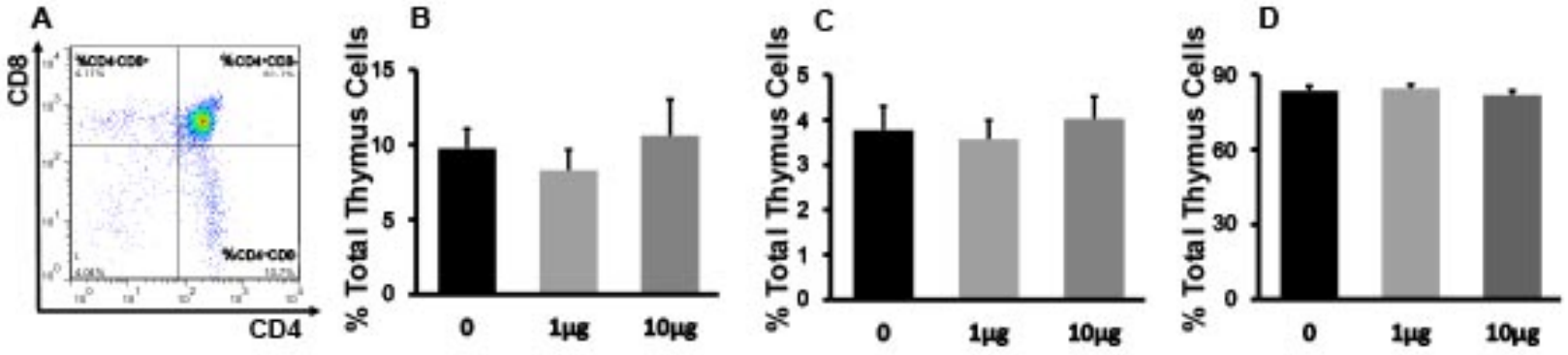

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Figure 3

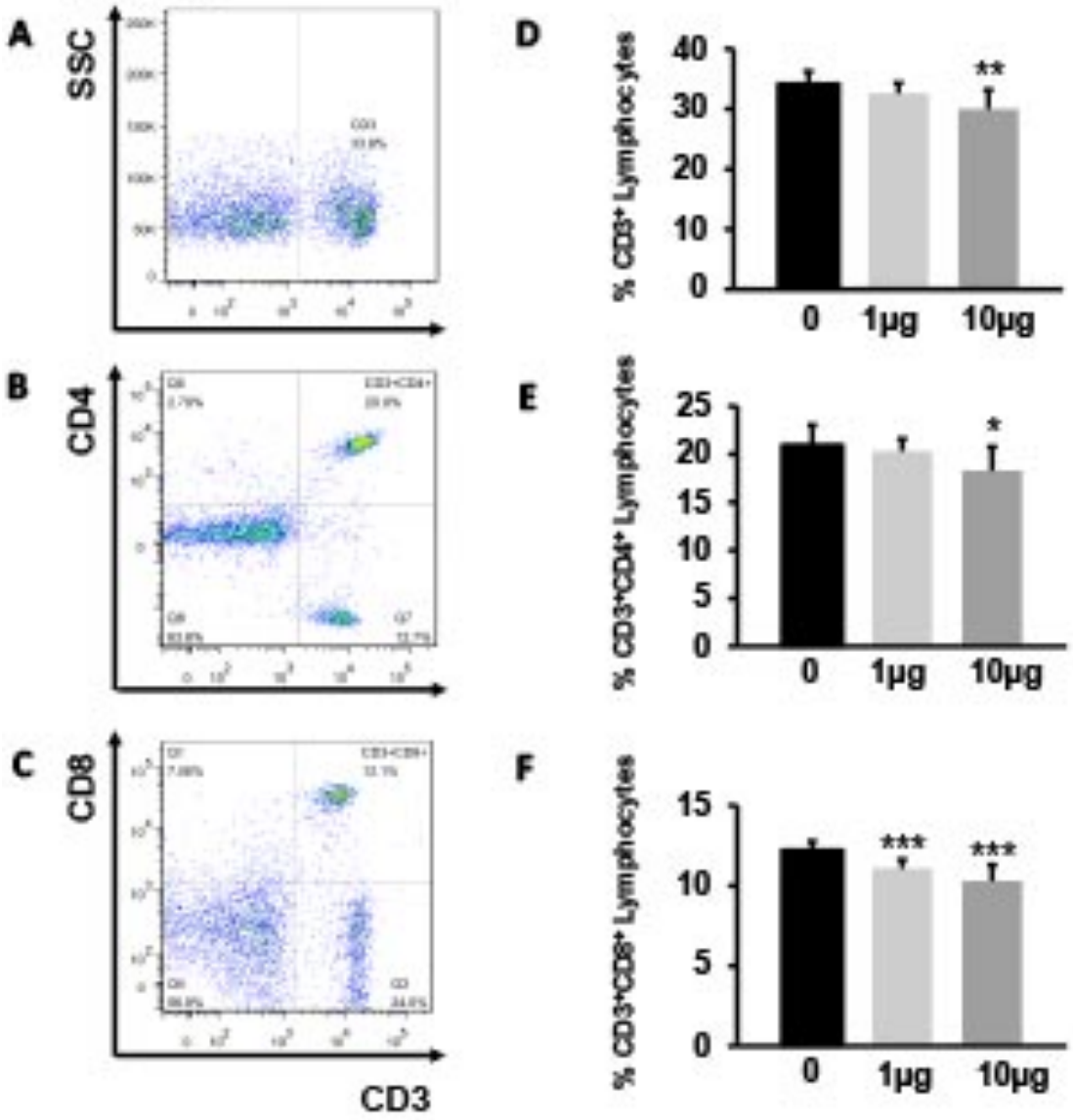

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Figure 4

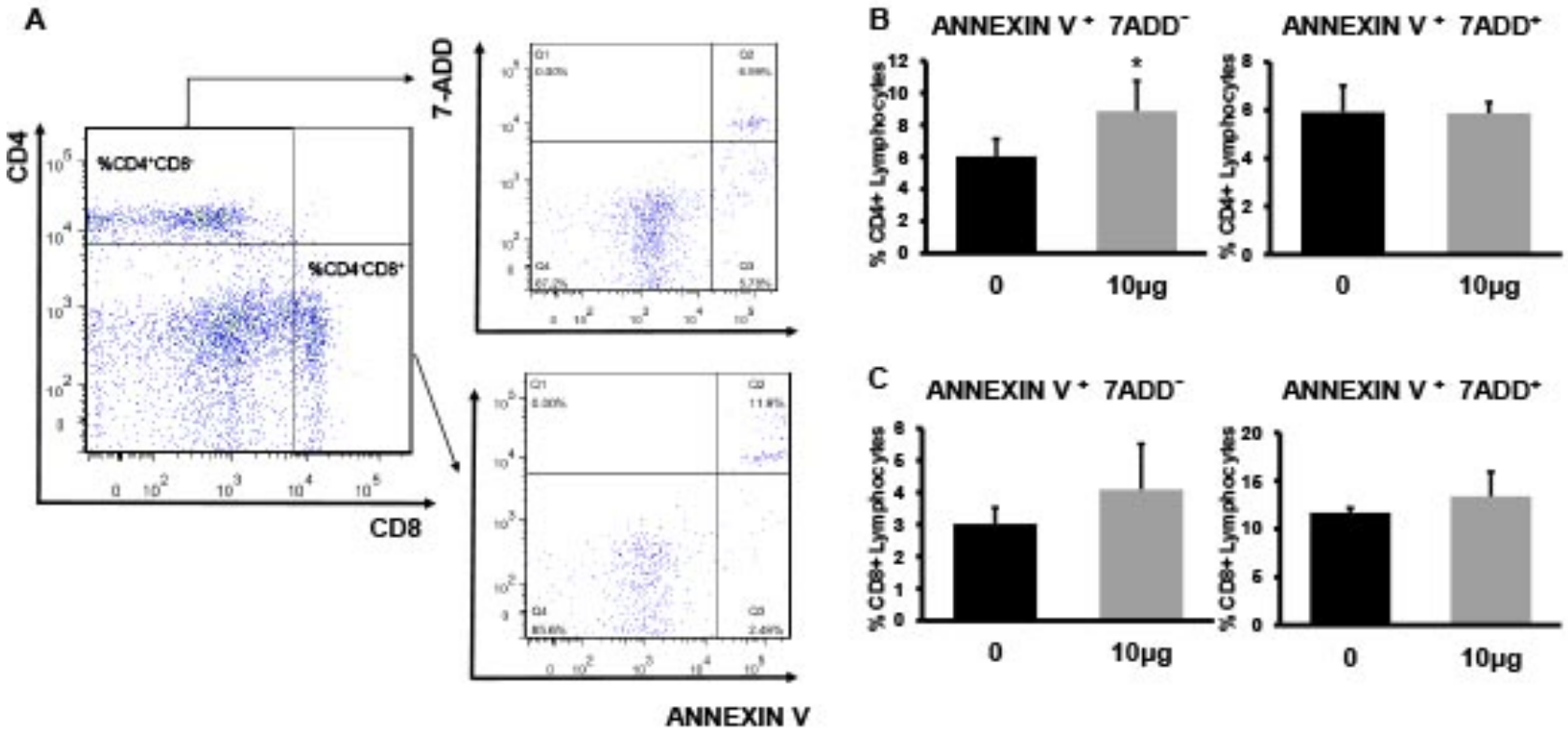

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Figure 5

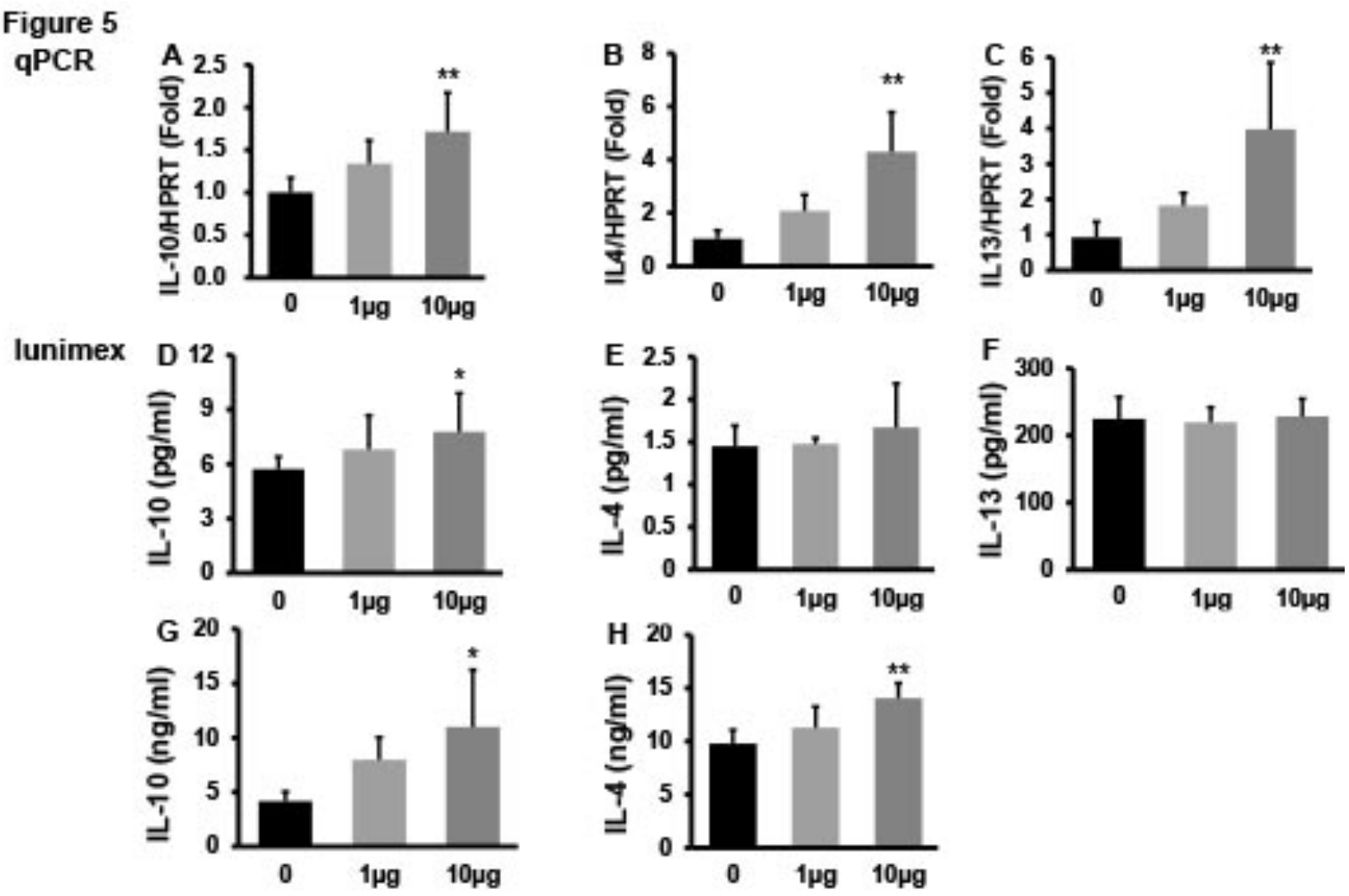

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Figure 6

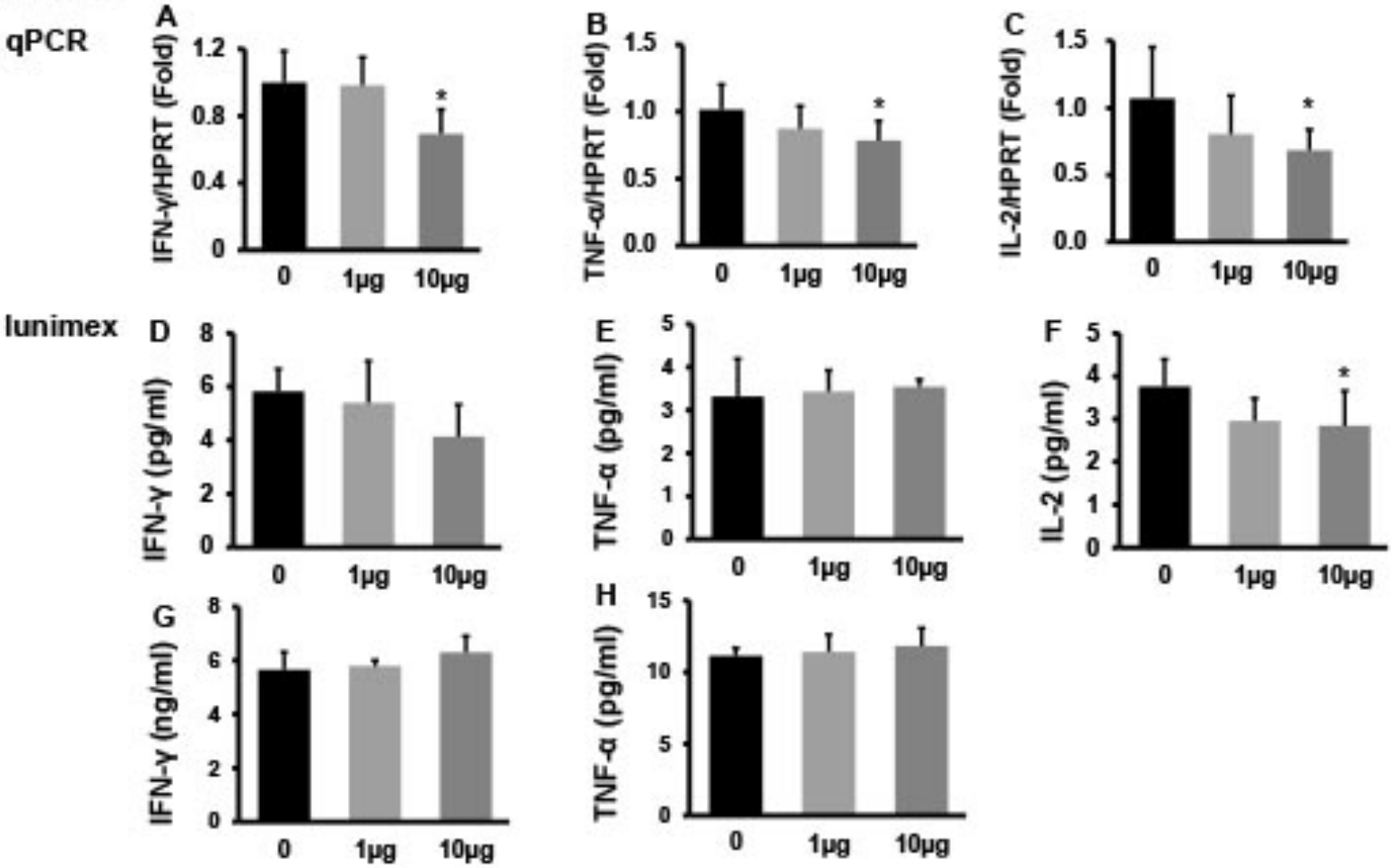

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Figure 7

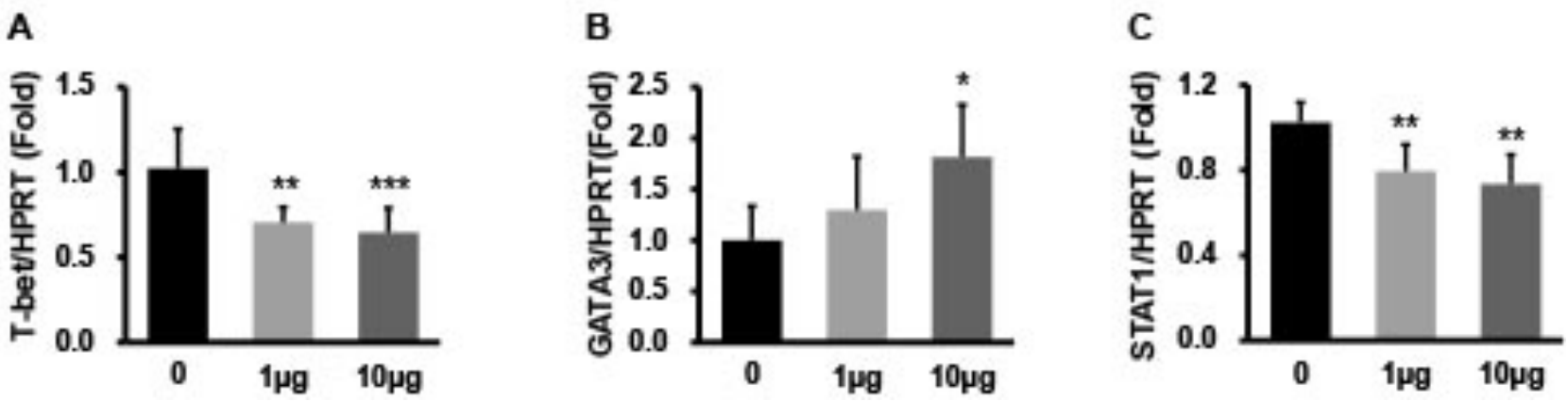

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Figure 8